performed in HeLa cells expressing MCU-V5 and Mfrn2-GFP. **Results**: In RLM, Ca^{2+} and Fe^{2+} (250 μM) each stimulated respiration to a nearly identical degree, an effect completely blocked by Ru360. In UMSCC22A cells, mRNA and protein expression of Mfrn2 was 2-3-times that observed in UMSCC1 cells. High Mfrn2-expressing UMSCC22 cells also had 3-fold greater rates of mitochondrial Ca^{2+} and Fe^{2+} uptake. After Mfrn2 knockdown (55% decrease), rates of mitochondrial uptake of both Ca^{2+} and Fe^{2+} decreased by ~75%. All uptakes were blocked by Ru360. In HeLa cells co-transfected with MCU-V5 and Mfrn2-GFP, anti-GFP beads pulled down MCU-V5, whereas anti-V5 beads pulled down Mfrn2-GFP. COX-IV was not pulled down by beads, indicating that the interaction between MCU and Mfrn2 was specific. **Conclusions**: 1) Mfrn2 positively modulates Ru360-sensitive respiration-driven mitochondrial uptake of both Ca^{2+} and Fe^{2+}. 2) Mfrn2 physically interacts with MCU and appears to be a component/regulator of the MCU complex. 3) The mitochondrial calcium uniporter should more appropriately be called the mitochondrial Ca^{2+},Fe^{2+} uniporter.

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K^+ Translocation by the Giant Axon of the Humboldt Squid Na^+/K^+ ATPase

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The Na^+/K^+ pump is a membrane protein which plays a fundamental role in maintaining the Na^+ and K^+ electrochemical gradients in animal cells. When internal and external Na^+ is absent the pump can only undergo K^+ translocation reactions. At equilibrium, the distribution of the different protein conformations depends on the rate constants of each step leading to K^+ binding and unbinding. If some of these rate constants are voltage-dependent, sudden changes in membrane electric potential will shift the binding-unbinding equilibrium. In those translocation reactions, K^+ has to travel a fraction of the membrane electric field generating a transient current signal. Here, K^+ pump currents were measured under voltage clamp conditions using the giant axon of the Humboldt squid, which due to its large diameter (1 -1.5 mm) allows the detection of these charge movements. By using H2DTG, a reversible inhibitor of the squid Na^+/K^+ pump, we were able to obtain H2DTG-sensitive transient currents in response to voltage jumps in K^+/K^+ conditions. Kinetics of these transient currents shows two main components, that in contrast to their Na^+ counterpart, appeared to be uncoupled. The origin of the fast component appears to be the movement of ions along an access channel that it is always open, suggesting that the gate that occlude K ions is deep in the permeation pathway. On the other hand, charge displacement distribution and rate constants of the slow component show a clear dependence on the K^+ external concentration revealing that the entrance of the K^+ to the Na^+/K^+ pump from the external side is a voltage-dependent step. Supported by FIRCA grant R03 TW008351 and U54GM087519, GM030376, NS64259, HL36783 and the Intramural Program of the NINDS/NIH and FONDECYT 1110430.

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Conformational Rearrangements of the Na^+/K^+ ATPase During Na^+ Occlusion/Deocclusion Transitions Assessed by Site-Directed Fluorescence

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The Na^+/K^+ ATPase is a E_1-E_2-type pump formed mainly by two subunits: α and β, and is responsible for Na^+ and K^+ homeostasis, thereby fundamental for cellular life. Most of the voltage dependence of the pump cycle originates from the steps associated with extracellular Na^+ binding and release, namely occlusion and deocclusion (E_1P and PE_2). To gain further insights into the conformational rearrangements of the squid Na^+/K^+ pump during the E_1P and PE_2 transitions, we have used site-directed flourometry under voltage-clamp. We engineered single cysteine mutants facing the outside of the α and β subunits of the squid Na/K pump, as targets of a cysteine-reactive fluorescent reporter (6-TMR). These constructs were found functional when expressed in *Xenopus* oocytes under voltage-clamp conditions and we obtained simultaneous electrical and fluorescence recordings. We have identified two positions on the α subunit: N894C (linker M7-M8) and D802C (linker M5-M6), and two on the β subunit: D74C (top of the TM helix) and D116C (external cap-helix), that produce voltage dependence fluorescence changes during the E_1P/PE_2 transitions. Fluorescence intensities produced by N894C, D74C and D116C constructs were correlated with its respective voltage dependent Na^+ translocation curves following a Boltzmann distribution. Interestingly, kinetics of the fluorescence and electrical signals from N894C showed remarkably similar voltage dependence, suggesting functional and conformational correlation during E_1P/PE_2 transitions. Surprisingly, fluorescence signal from D116C produced a biphasic kinetics behavior, indicating a complex movement of the β and/or the α with respect to the β subunit. These findings begin to delineate a region in the α-subunit that is moving during the E_1P/PE_2 transitions. Supported by U54GM087519 and GM030376.

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State-Dependent Movement between the First and Last External Loops of the Na/K Pump α Subunit

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Previously we reported that conserved Na/K pump α-subunit residues D128 (loop L1-2, shark-α1 numbering) and R979 (L9-10) reach atomic proximity in E2P (Artigas, 2009. *Biophys J.* 96(3):145a). L1-2 is expected to move throughout the pump cycle. We used double cysteine substitutions to address the relative displacement of L1-2 with respect to R979. We mutated R979C and concomitantly introduced a cysteine at each of the residues within L1-2, from Q118 (external end of TM1) to L132 (within TM2), and analyzed the functional effect of reducing and oxidizing reagents on these double cysteine mutants heterologously expressed in *Xenopus* oocytes. The pump current (I_P) induced by 3 mM K under two-electrode voltage clamp in the absence of Na was measured; first without redox treatment, then after 15 min in 10 mM TCEP (a reducing agent) and subsequently, following oxidation with cupper phenanthroline (100 μM Cu: 300 μM Phe, applied in N-Methyl D-glucamine). TCEP increased the I_P of E122C/R979C (20%), E124C/R979C (20%), P125C/R979C (38%), Q126C/R979C (52%) and D128C/R979C (130%), whilst Cu:Phe induced similar effects, in the opposite direction than TCEP, with a maximum reduction of I_P (>80%) in D128C/R981C. The effect of Cu:Phe on the charge movement without K in the presence of Na was also studied. Cu:Phe abolished charge movement of D128C/R981C and modified the characteristics of the transients of the other double cysteine mutants that showed effects of crosslinking in I_P. Our results indicate that residue 128 (at the end of a rigid TM2-helix) must separate from R979 in order for TM2 to perform its required motions, while crosslinking residues further in the flexible loop does not block I_P, but modifies the E1-E2 conformational equilibrium in the presence of Na. Supported by R15NS081570-01A1.

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Single-Molecule Measurements to Investigate the Negative Cooperativity in Na^+/K^+ -ATPase

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The Na^+/K^+-ATPase, a cell membrane ion motive ATPase, uses energy from the hydrolysis of ATP to move Na^+ out of and K^+ into cells, thus maintaining the membrane resting potential and cellular volume. To investigate how this pump functions, we isolated ATPase from duck supraorbital salt glands and labeled it with Cy3-maleimide (Cy3-ATPase). In bulk experiments, we found that the fluorescence of Cy3-ATPase decreases in the presence of ATP (*Biochim Biophys Acta* 2009; 1794:1549-1557). The kinetics of this ATP-induced fluorescence decrease exhibited negative cooperativity and could be explained in terms of protein aggregation. To further explore the phenomenon of negative cooperativity on the level of individual monomers, we used single-molecule total internal reflection fluorescence (SM-TIRF) microscopy. Protein monomers were solubilized and reconstituted into lipid vesicles to investigate the effect of varying ATP concentration on the fluorescence.
Data from SM-TIRF experiments, analyzed using a hidden Markov model (HMM), suggest that the Cy3-ATPase exists in dynamic equilibrium between a high fluorescence state (unquenched) and a low fluorescence state (partially quenched). These kinetics are characterized by either rapid or slow transitions between these states. Two subpopulations are observed, one where the transitions between the states occur rapidly and the other where the kinetics are slower. Preliminary analysis of the data suggests that ATP shifts the population distribution from those exhibiting rapid transitions to those exhibiting slow transitions. Here, we report on the analysis of these effects and the implications of the above observations on the working of the pump.

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The Molecular Mechanism of Na^+, K^+-ATPase Malfunction in Mutations Characteristic for Adrenal Hypertension

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Mutations within ion transporting proteins may severely affect their ability to properly traffic ions and thus perturb the delicate balance of ion gradients.

Somatic gain-of-function mutations of the Na^+, K^+-ATPase ion pump α1-subunit have been found in aldosterone-producing adenomas that are amongst the causes of hypertension. We use all-atom Molecular Dynamics (MD) simulations to investigate structural consequences of these mutations, namely Leu97 substitution by Arg (L97R), Val325 substitution by Gly (V325G), deletion of Phe93, Ser94, Met95, Leu96, Leu97 (Del93-97) and deletion-substitution of Glu953, Glu954, Thr955, Ala956 by Ser (EETA956S) that show inward leak currents under physiological conditions. First three mutations affect the structural context of the key ion binding residue Glu327 at binding site II, which leads to the loss of the ability to correctly bind ions and to occlude the pump. The mutated residue in L97R is more hydrated, which ultimately leads to the observed proton leak. V325G mutant mimics the structural behavior of L97R, however it does not promote the hydration of surrounding residues. In Del93-97, a broader opening is observed due to the rearrangement of the kinked transmembrane helix 1, M1, which may explain the sodium leak measured with this mutant. The last mutant, EETA956S, opens an additional water pathway near the C-terminus, affecting the III sodium-specific binding site. Moreover, we report for the first time the spontaneous binding of monovalent ions to the E2P outside open state of the Na^+, K^+-ATPase. The results support electrophysiology measurements and suggest how three mutations prevent the occlusion of the Na^+, K^+-ATPase, with a possibility of transforming the pump into a passive ion channel, while the fourth mutation provides a new insight into the sodium binding in the E1 state.

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Glutathionylation of the Na K Pump

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Reversible oxidative modifications of proteins are of importance for the normal cell functioning, e.g. for the receptor-coupled signaling. The formation of a disulfide bond between a protein and a glutathione tripeptide (Glu-Cys-Gly,) is an oxidative, posttranslational modification that might be involved in the cellular signaling. Recently, the Cys46 residue of the sarcolemmal Na K ATPAse β1 subunit has been proposed as a target for glutathionylation. Here, we use all-atom Molecular Dynamics (MD) simulations to investigate structural consequences of the Cys46 glutathionylation in the E2P state of the protein. Being negatively charged at physiological pH, the glutathione modification can induce alterations similar to the effect of phosphorylation. In contrast with previous studies, we find that Cys46, buried deeply in the membrane, can be exposed to the cytosolic glutathione due to the defect and local rearrangement of the protein-membrane interface in the E2P state, rather than the β1 helix sliding outside the membrane, previously anticipated for the E1 state. These findings are in accord with the recent crystal structure of the E1P state of Na^+, K^+-ATPase, where the position of the β1 helix is essentially the same as it is in the E2P state. We will comment on the accessibility of glutathione to Cys46, which lies at the center of the membrane.

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Superinhibitory Phospholemman Mutants as Potential Therapeutics for Heart Failure

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The sodium-potassium ATPase (NKA) is a target for inotropic cardiac glycosides that inhibit NKA activity and cause a downstream increase in intracellular calcium. These agents have a narrow therapeutic window and can cause arrhythmias. In contrast, the endogenous inhibitor of NKA, phospholemman (PLM), is dynamically regulated. Inhibition of NKA is relieved by elevated intracellular sodium, or when PLM is phosphorylated. In the present study, we performed scanning alanine mutagenesis of PLM to identify superinhibitory mutants of PLM that could serve as an alternative to cardiac glycosides. We hypothesized that mutations that destabilize PLM oligomers will also cause increased binding of PLM to NKA. We employed a FRET assay that simultaneously reports PLM-PLM and PLM-NKA binding for each mutant. Several mutants conformed to this prediction, showing decreased oligomerization and higher affinity for NKA compared to WT. Interestingly, other mutants demonstrated greater affinity for NKA despite no detectable decrease in oligomerization. Ongoing studies will determine whether high affinity PLM mutants function as superinhibitors of NKA activity.

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Molecular Dynamics Simulations Helps to Rationalize CopB Mutations and their Relationships to Wilson Disease

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The regulation of copper levels is central to physiology. Mutations in the ATP7B copper transporter are known to lead to Wilson's disease in humans. How these mutations lead to the disease is not fully characterized at a molecular level. An excellent model system for exploring the changes in structure and dynamics for Wilson disease mutations for the ATP binding domain is provided by CopB from A. fulgidus. This protein has high sequence similarity with the P, N and hinge regions of ATP7B. Mutations to each region have previously been characterized by experimental measurements. In this presentation we highlight implicit and explicit solvent simulations of the wild-type and mutations found within each of these three regions. The results shed new light on how the mutations impact on conformational change, on ATP-binding, and on phosphorylation within these domains.

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Peptide-Based Approach to Study Cytosolic Domain Interactions in a Bacterial Copper-Transporting ATPase

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Copper-transporting P_{IB}-type ATPases (CopA-family) fulfill key roles in copper homeostasis by pumping copper across bacterial or intracellular and cytoplasmic eukaryotic membranes. Mutations of the copper-transporting ATPase gene cause Wilson and Menkes diseases. CopA consists of 8 transmembrane helices and three cytosolic domains; Nucleotide binding (N), Phosphorylation (P) and Actuator (A) domains. We have used synthetic peptides to identify minimal structural motifs and physical mechanisms of cytosolic domain interactions.

Based on the crystal structure[1], we have designed decameric-peptides whose sequences are derived from the putative interaction site of the A-domain with the PN domain of CopA from *Legionella pneumophila.* The interaction of the synthetic A-domain peptide with the individually expressed PN domain was measured by Isothermal Titration Calorimetry(ITC), Circular Dichroism (CD), and stopped-flow fluorescence techniques. Preliminary ITC results show a favorable TΔS, indicating that binding of the peptide carrying the native sequence is driven by hydrophobic interactions with stoichiometry(N=1). Time-resolved fluorescence measurements with a tryptophan-carrying actuator peptide show association and dissociation rate constants with the PN domain of $22000M^{-2}s^{-1}$ and $0.7M^{-1}s^{-1}$, respectively. This corresponds to a K_d of the peptide for the PN-domain of 32μM. In the presence of the non-hydrolyzable nucleotide AMPPNP the best kinetic fit was obtained with rates of $20000M^{-2}s^{-1}$ and $1.0M^{-1}s^{-1}$, indicating an almost unaffected affinity of the peptide for the nucleotide-loaded PN-domain. In agreement with the kinetic and isothermal data, the decapeptide also shifted the melting temperature of the PN domain from 330 to 332K as monitored by CD spectroscopy.

In summary, we have good evidence that the actuator peptide binds in a predominantly hydrophobic mechanism to the PN domain in the 30-50μM K_d range with little dependence on the occupancy of the nucleotide binding site of the PN domain.

1)Gourdon,P. et.al.2011 Nature475, 59.

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Conformational Transitions in ATP-Driven Calcium Pump SERCA

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Sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) is an integral membrane protein that uses ATP hydrolysis as a source of free energy to pump two calcium ions per ATP molecule from calcium poor cytoplasm of the muscle cell to the calcium rich lumen of the sarcoplasmic reticulum, thereby maintaining a ten thousand fold concentration gradient. Detailed structural studies of the pump under different conditions provided analogues of various intermediates in the reaction cycle and revealed important changes in the tertiary structure of the protein both in the cytoplasmic and in the